

PERSISTENCE OF *SALMONELLA* TYPHIMURIUM IN PORCINE GUT MICROFLORA

Timothy J. Anderson, Toni L. Poole, Robin C. Anderson*, David J. Nisbet

*United States Department of Agriculture, Agricultural Research Service, Food & Feed Safety Research Unit, College Station, TX, 77845, USA, Ph: 01-979-260-9317, Email: anderson@ffsru.tamu.edu

Abstract Chlortetracycline administration, at 55 mg/l, to a continuous flow culture of mixed porcine gut bacteria enhanced the rate of clearance of a chlortetracycline resistant *Salmonella* Typhimurium from the culture, although the *Salmonella* was eventually excluded from the culture by 8 days post challenge. As expected, chlortetracycline administration, at 110 mg/l, to a continuous flow culture of mixed porcine gut bacteria had little effect on the persistence of a chlortetracycline resistant strain of *Salmonella* Typhimurium. The competitive fitness of this resistant strain was enhanced even during culture in the absence of chlortetracycline, thus indicating that even without potentially selective effects of chlortetracycline on the continuous flow culture's resident flora, the resistant *Salmonella* was more competitive than its sensitive *Salmonella* counterpart.

Introduction Feed additives have been used in the animal industry for over three decades to improve growth rate, feed utilization and reduce both morbidity and mortality from bacterial infections (Dritz *et al*, 1997). The wide spread use of antibiotics as feed additives; however, has raised concerns that a reservoir of antibiotic resistant bacteria may become established in the animal and its environment. Whereas the ability of a healthy gut microflora to exclude enteropathogens in the absence of antibiotics has been widely reported, much less is known regarding the gut flora's exclusion potential when in the presence of an antibiotic, especially when the competing pathogen expresses resistance to the administered antibiotic. The purpose of the present study was to determine the fitness of chlortetracycline sensitive and resistant *Salmonella enterica* serovar Typhimurium during competition in a mixed population of porcine gut bacteria grown in continuous flow culture with or without chlortetracycline exposure.

Materials and Methods Continuous flow cultures of *Salmonella* Typhimurium were established with a mixed population of porcine cecal bacteria as previously described (Harvey *et al*, 2002; Hume *et al*, 2001). Briefly, 1,150-ml chemostat vessels containing 1,050 ml of Viande Levure (VL) broth (Barnes *et al*, 1979) were inoculated with cecal contents collected from a healthy pig (Hume *et al*, 2001) and incubated anaerobically at 39°C. The continuous flow cultures were operated at a dilution rate of 0.0416 per hour which corresponds to a flow rate of 0.80 ml/min and a vessel turnover time of 24 h. Continuous flow cultures of mixed cecal bacteria reached steady state after approximately 14 vessel turnovers at which time 100 ml of *Salmonella* Typhimurium (of pig origin) grown overnight at 37°C in Typtic Soy Broth broth was added.

For treated cultures, chlortetracycline (55 or 110 mg/l) was administered to both the medium reservoir and chemostat vessel immediately after *Salmonella* addition. Samples taken from the mixed culture chemostats at the times indicated were serially diluted (10-fold) in sterile phosphate buffered saline (PBS, pH 7.0) and plated on Brilliant Green Agar (BGA) (Oxoid, Unipath LTD., Basinstoke, Hampshire, UK) and incubated at 37°C overnight. All experiments were performed in duplicate and means from each treatment were analyzed for differences using a Student's *t* test with PC-SAS version 6.02 Statistical Software (SAS Institute Inc., Cary, NC, USA).

Results Concentrations of a chlortetracycline sensitive *Salmonella* Typhimurium varied little immediately after addition to the mixed culture chemostats and were reduced to undetectable levels (< 10 CFU/ml) in the mixed culture chemostat by 8 days of culture whether cultured in the absence or presence of 55 mg/l chlortetracycline (Figure 1). However, when cultured in the presence of chlortetracycline, the sensitive *Salmonella* was reduced to a significantly greater degree ($P \leq 0.05$) for each day from day 2 through day 7 when compared to that of the control. Consequently, this sensitive *Salmonella* strain was reduced to undetectable levels 3 days faster when grown in the presence of 55 mg/l chlortetracycline than when grown without the antibiotic treatment. Chlortetracycline resistant *Salmonella* Typhimurium were not detected throughout the course of this ten day study.

To determine if antibiotic resistance offers a bacterium a selective advantage over the endogenous microflora under conditions of antibiotic selection, a chlortetracycline resistant *Salmonella* Typhimurium was added to mixed culture chemostat and 110 mg/l chlortetracycline was added to the culture medium and chemostat vessel. Over the first six days, this resistant *Salmonella* was excluded at almost the same rate during culture in both the treated and untreated chemostats (Figure 2). On days 7 through 14, concentrations of chlortetracycline resistant *Salmonella* Typhimurium were lower ($P \leq 0.05$) in the chemostats treated with 110 mg/l chlortetracycline than in untreated control vessels.

Discussion/Conclusion Presently, we report results from a study modeling the competitive fitness of chlortetracycline sensitive or resistant *Salmonella* Typhimurium during continuous flow culture in mixed populations of commensal pig gut bacteria. These model populations are particularly well suited for studying biological processes occurring within complex and dynamic ecosystems because they offer an effective yet affordable way to simulate competitive processes that occur in natural habitats (Harvey *et al*, 2002; Hume *et al*, 2001; Nisbet *et al*, 2000). We found that the addition of chlortetracycline at a level approximating its use as a growth promoter caused a rapid decrease in *Salmonella* Typhimurium over the first 5 days as compared to untreated control cultures; however, in both treated and untreated cultures *Salmonella* Typhimurium was reduced to undetectable levels by day 8. This suggests that chlortetracycline was effective against this sensitive *Salmonella* but that even without antibiotic treatment, the *Salmonella* strain was effectively eliminated by the cultures resident microflora. The mixed culture received a single challenge by *Salmonella* in this study; however, whereas under natural conditions of exposure it would be expected that animals may be chronically exposed to *Salmonella* Typhimurium via contaminated feed, water or from animals whose normal microflora has been disrupted through disease or other mitigation. Contrary to that observed with a human volunteer exposed to tetracycline, where resistant colonies from feces peaked within 2 days after exposure (Levy, 1997), chlortetracycline resistant *Salmonella* Typhimurium were not detected over the course of our first study. It is not clear, however, whether the development of resistance did not occur or that our cultural conditions were not conducive for recovery of chlortetracycline resistant *Salmonella*, which may have otherwise been compromised in their competitive fitness.

Under conditions of antibiotic selection it would be expected that antibiotic resistant bacteria would have a selective advantage over the normal susceptible microflora. In our second study, chlortetracycline appeared to provide little, if any, direct selective advantage to a resistant *Salmonella* Typhimurium as concentrations of bacterium appeared to diminish within the continuous flow cultures at nearly equal rates regardless of whether cultured in the presence or absence of chlortetracycline. However, even in the absence of chlortetracycline, and thus in the absence of antibiotic pressure against the resident microflora, the resistant *Salmonella* Typhimurium appears to have been more competitive than that observed with the sensitive *Salmonella* Typhimurium in the first study. For instance, the resistant *Salmonella* persisted longer, for at least 14 days, and at concentrations 10 to 100-fold higher in the mixed pig flora populations. This suggests that acquisition of compensatory mutations in this chlortetracycline resistant *Salmonella* strain may have conferred competitive fitness independent of resistance to added chlortetracycline.

References

- Barnes, E.M., Impey, C.S., Stevens, B.J.H., 1979. Factors affecting the incidence of and anti-salmonella activity of the anaerobic cecal flora of the young chick. *J of Hygiene* 82:263-283
- Dritz, S.S., Tokach, M.D., Goodband, R.D., Nelssen, J.L., 2002. Effects of administration of antimicrobials in feed on growth rate and feed efficiency of pigs in multisite production systems. *J Am Vet Med Assoc* 220:1690-1695
- Harvey, R.B., Droleskey, R.E., Hume, M.E., Anderson, R.C., Genovese, K.J., Andrews, K., Nisbet, D.J., 2002. *In vitro* inhibition of *Salmonella enterica* serovars Choleraesuis and Typhimurium, *Escherichia coli* F-18, and *Escherichia coli* O157:H7 by a porcine continuous-flow competitive exclusion culture. *Curr Microbiol.* 45:226-229.

Hume, M.E., Nisbet, D.J., Buckley, S.A., Ziprin, R.L., Anderson, R.C., Stanker, L.H., 2001. Inhibition of in vitro *Salmonella* Typhimurium colonization in porcine cecal bacteria continuous-flow competitive exclusion cultures. *J Food Prot.* 64:17-22.

Levy, S.B., 1997. Antibiotic resistance: an ecological imbalance. In *Antibiotic Resistance: Origins, Evolution, Selection and Spread*, edited by DJ Chadwick, J Goode. West Sussex, England: Wiley, Chichester (Ciba Foundation Symposium 207), pp 1-14.

Nisbet, D.J., Anderson, R.C., Corrier, D.E., Harvey, R.B., Stanker, L.H., 2000. Modeling the survivability of *Salmonella* Typhimurium in the chicken cecae using an anaerobic continuous-culture of chicken cecal bacteria. *Microbial Ecol Health Dis.* 12:42-47.

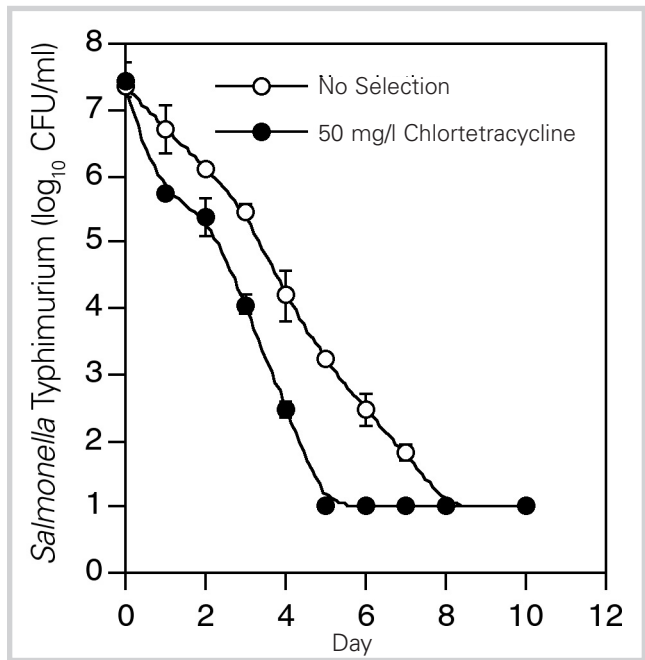


Figure 1. Clearance of chlortetracycline sensitive *Salmonella* Typhimurium from a mixed population of porcine cecal bacteria cultured in continuous flow culture in the absence or presence of chlortetracycline treatment. Open circles indicate cultures with no selection, filled-in circles indicate cultures treated with 55 mg/l chlortetracycline. Error bars indicate standard deviation.

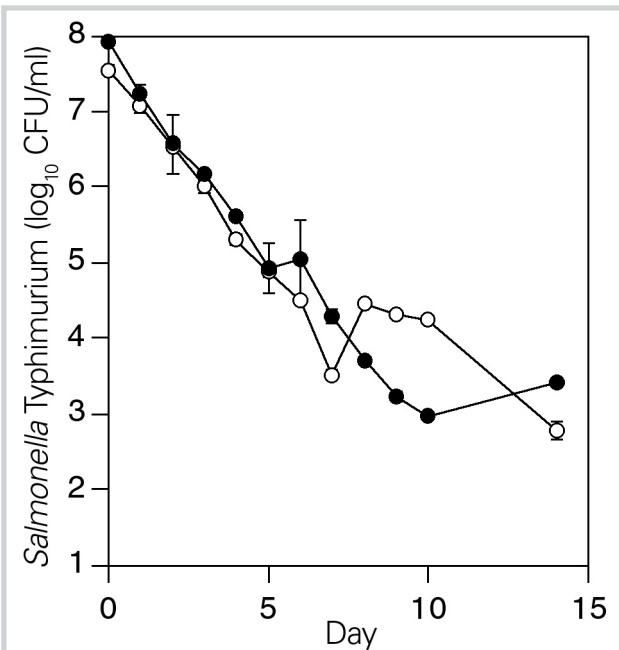


Figure 2. Clearance of chlortetracycline resistant *Salmonella* Typhimurium from a mixed population of porcine cecal bacteria cultured in continuous flow culture in the absence or presence of chlortetracycline treatment. Open circles indicate cultures with no selection, filled-in circles indicate cultures treated with 110 mg/l chlortetracycline. Error bars indicate standard deviation.